New genotypes of TT virus (TTV) and a genotyping assay based on restriction fragment length polymorphism

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Abstract A phylogenetic analysis, using the open reading frame 1 sequence of 93 TT viruses (TTV) obtained from various geographical areas, indicated that the virus could be classified into six different genotypes including three hitherto unreported genotypes. The high reliability of the six clusters was confirmed by bootstrap analysis. On the basis of these sequence data, a new simple genotyping assay based on a restriction fragment length polymorphism of TTV was developed. Using the enzymes *Nde*I and *Pst*I, followed by cleavage with *Nla*III or *Mse*I, it was possible to distinguish between the six TTV genotypes. This system will provide the framework for future detailed epidemiological and clinical investigations.

Key words: Genotype; Phylogenetic analysis; Restriction fragment length polymorphism; TT virus

1. Introduction

Recently, a new virus was isolated from the serum of a patient with posttransfusion hepatitis of unknown etiology, using representational difference analysis [1]. This virus, designated as the TT virus (TTV), is a single-stranded DNA virus, similar to the human parvovirus B19 [2]. TTV DNA was detected in 12% of healthy blood donors, although the serological prevalence of TTV infection in healthy blood donors was lower than that in patients with fulminant or chronic cryptogenic liver diseases [2]. Recent analyses, based on a phylogenetic tree constructed using the open reading frame (ORF) 1 sequence of TTV, showed that the virus could be classified into three different genotypes, namely, genotype 1 (G1), genotype 2 (G2) and genotype 3 (G3), and that the genetic distances between the three genotypes were very great [3]. In Japan, G1 was the most common genotype of TTV [2]; however, it is still unclear whether any correlation exists between the TTV genotypes and their geographical distribution or pathogenicity.

In this study, our evolutionary analysis using several TTV isolates revealed the existence of six different genotypes of TTV, that is, three other genotypes in addition to the three hitherto reported genotypes, tentatively named genotype 4 (G4), genotype 5 (G5) and genotype 6 (G6) [3]. A new simple genotyping assay based on restriction fragment length polymorphism (RFLP) of TTV was designed. We reported six different genotypes of TTV by phylogenetic analysis and the new genotyping assay based on RFLP.

2. Materials and methods

2.1. Subjects

Seventy-two serum samples obtained from Asia, Africa and South America, which were positive for TTV DNA as determined by the semi-nested polymerase chain reaction (PCR) using primers derived from ORF 1 [2], were used for this study, after obtaining informed consent. The samples were collected from subjects with liver diseases and healthy blood donors.

2.2. Methods

2.2.1. Detection of TTV DNA. Serum samples immediately after collection were stored at −80°C until assayed. Serum DNA was extracted from 200 μl serum using the High Pure Viral Nucleic Acid Kit with proteinase K (Boehringer Mannheim, Penzberg, Germany), and TTV DNA was detected by semi-nested polymerase chain reaction (PCR) with Perkin-Elmer AmpliTaq Gold DNA Polymerase (Roche Molecular Systems, Branchburg, NJ, USA). The specific primers of TTV used for the PCR were kindly provided from Dr. Okamoto, Jichi Medical School, Tochigi, Japan [2]. In brief, the first round of PCR was performed with the sense primer NG059 and the anti-sense primer NG063 for 9 min at 96°C, followed by 35 cycles consisting of denaturation for 30 s at 94°C, annealing for 45 s at 60°C, and extension for 45 s at 72°C, in a 96-well cycler (GeneAmp 9600, Perkin-Elmer Cetus, Norwalk, USA). The second round of PCR was performed with the sense primer NG061 and the anti-sense primer NG063 for 25 cycles, under the same conditions as used for the first round of PCR.

The amplicons were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide, and observed under ultraviolet light. To confirm the specificity of amplification, the nucleotide sequences of the amplicons were determined by direct sequencing of the amplified products with a 373A DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.2.2. Molecular evolutionary analysis. Molecular evolutionary analyses were performed to investigate the similarity between the isolates in this study and previously reported TTV strains from Japan and Europe [1–3]. Using the computer program ODEN version 1.1.1. [4], the number of nucleotide substitutions per site and the genetic distances between the isolates were estimated by the 6-parameter method [5]. Based on these values, a phylogenetic tree was constructed by the neighbor-joining (N-J) method [6]. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times [7].

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All nucleotide sequence data reported in this paper have now been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases.

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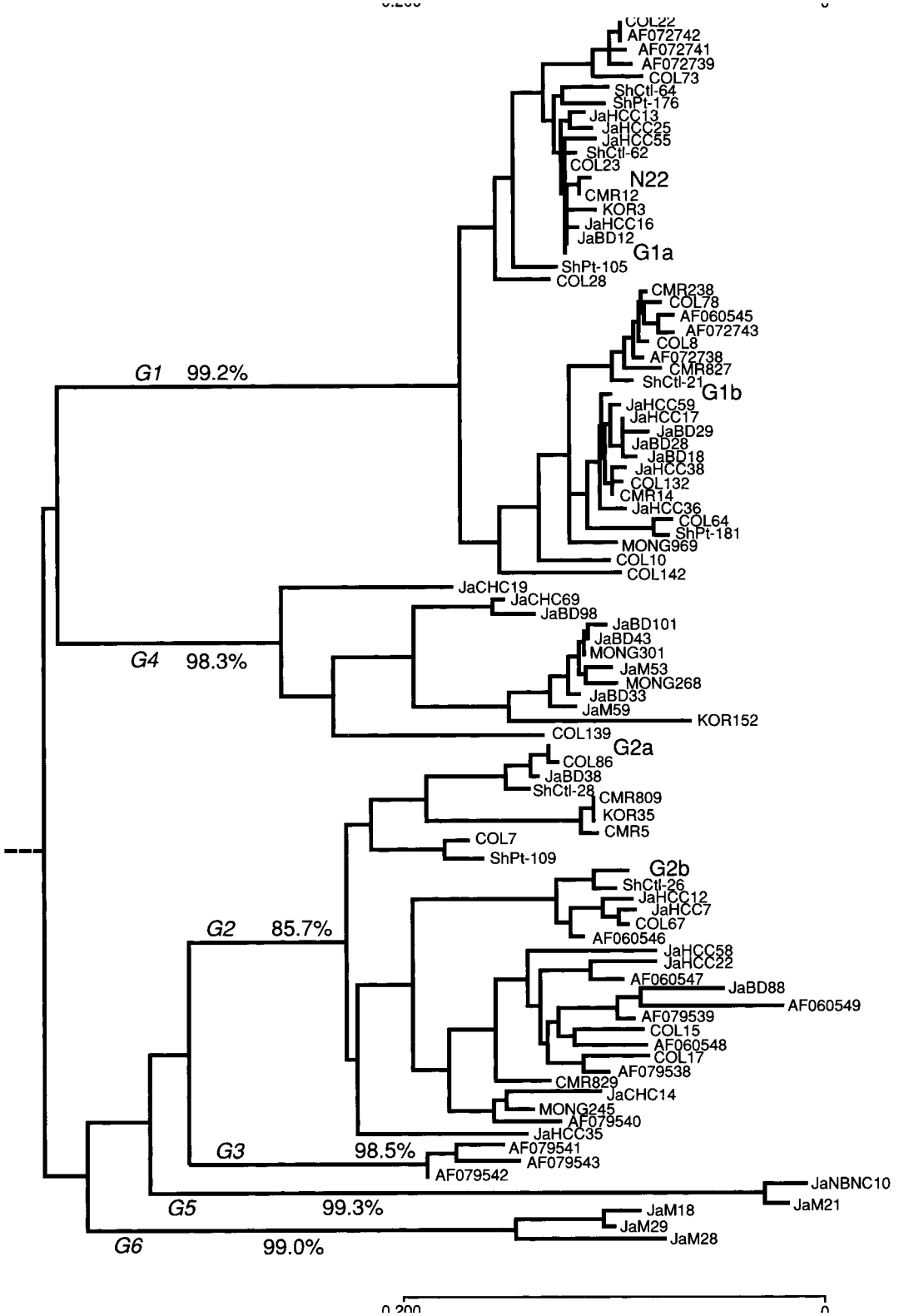

Fig. 1. Phylogenetic tree on the basis of the TT virus partial open reading frame 1 (ORF1) sequence as constructed by the neighbor-joining (N-J) method. Note that there are six major clusters, tentatively named G1, G2, G3, G4, G5, and G6. On bootstrap analysis for evaluation of the statistical reliability of the tree, the G1, G2, G3, G4, G5, and G6 clusters exhibited 99.2%, 85.7%, 98.5%, 98.3%, 99.3% and 99.0% reliability, respectively. The horizontal bar indicates the number of nucleotide substitutions per site. Ja = Japan, COL = Colombia, CMR = Cameroon, Sh = Shanghai, KOR = Korea, MONG = Mongolia, BD = blood donor. N22, G1a, G1b, G2a, and G2b were obtained from Japan [2]. AF060545–AF060549 were obtained from Germany, and AF072738–AF072743 and AF079538–AF079543 were obtained from the United Kingdom [3].

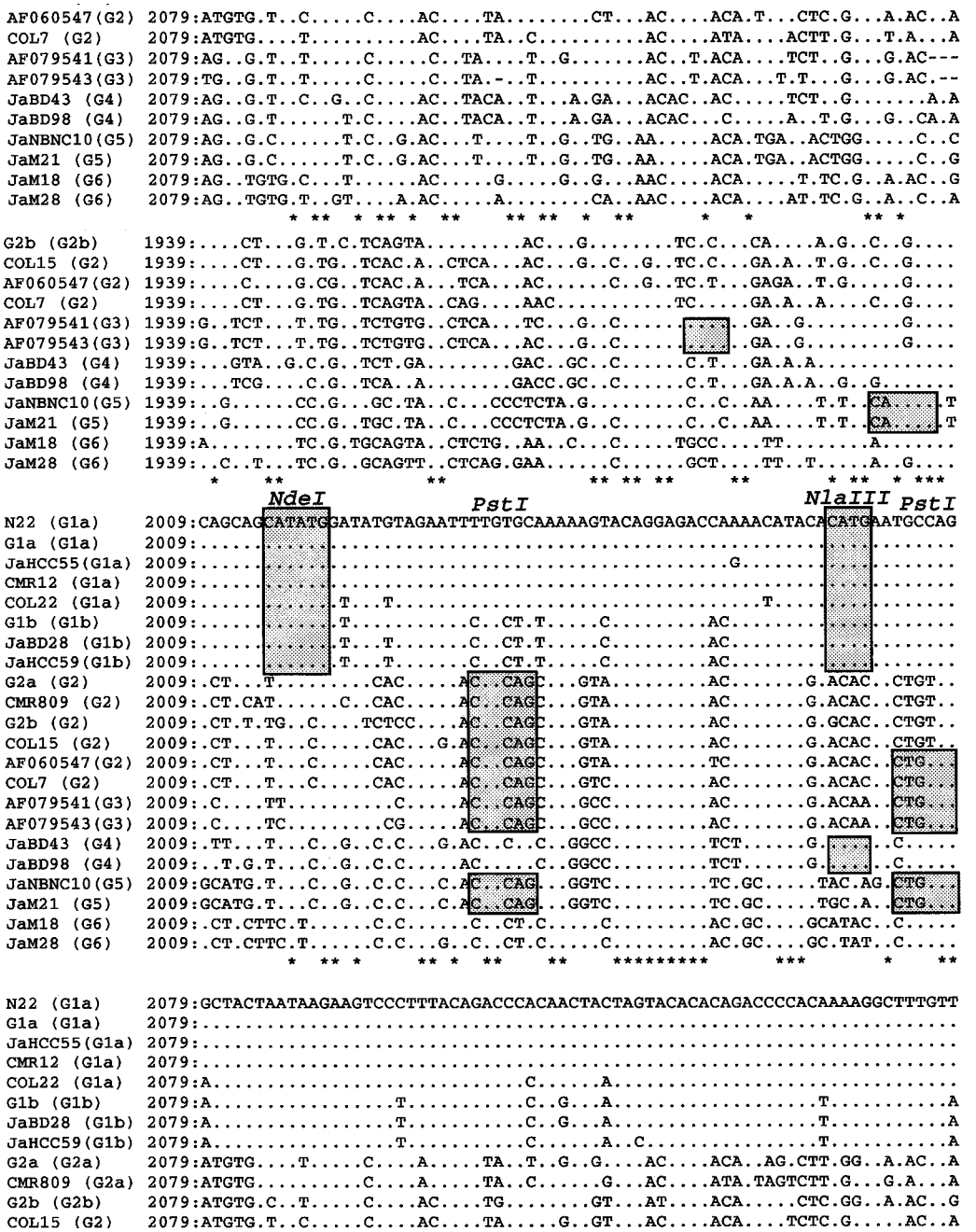

Fig. 2. Alignment of the ORF 1 sequences of the TTV isolates. These nucleotide sequences were derived from each of the six genotypes. The prototypes N22, G1a, G1b, G2a, G2b [2] and AF079541, AF079543 [3] were also included for comparison. The consensus nucleotide sequences (N22) of these isolates are given in the top line. The restriction sites recognized by the restriction enzymes *Nde*I, *Pst*I, *Nla*III, and *Mse*I are boxed. Identical nucleotides are shown as dots.

2.2.3. RFLP analysis. A new genotyping assay, based on RFLP analysis, was developed. The alignment of sequences determined as above revealed the presence of genotype-specific restriction sites, combinations of which determined each genotype as shown in Fig. 2. Restriction digestions were carried out with 10 μl of the second round PCR products for 3 h after adjustment with 10× enzyme reaction buffer according to the manufacturer's instructions. Reactions were carried out with 10 units of *Nde*I, *Pst*I (New England BioLabs, MA,

USA), or *Nla*III (Toyobo, Osaka, Japan) at 37°C. The digested PCR products were electrophoresed on 3% agarose gels, stained with ethidium bromide. The RFLP pattern was then evaluated under ultraviolet light.

3. Results

The nucleotide sequences of all the 72 TTV isolates in this study were aligned wiht N22, TA278 (G1a), TX011 (G1b), TS003 (G2a) and NA004 (G2b), obtained from Japanese subjects [2], and some European isolates obtained from DDBJ/EMBL/GenBank DNA databases. When a phylogenetic tree was constructed on the basis of a partial open reading frame sequence (222 bp – excluding primer sequences) using the N-J method (Fig. 1), the 93 sequences could be classified into six major genotypes. In brief, 43 iolates belonged to G1, 30 to G2, 3 to G3 [3], and 17 were classified into three hitherto unreported genotypes, which were tentatively named G4, G5, and G6; 12 isolates belonged to G4, 2 to G5 and 3 to G6. The major genotypes, G1 and G2, were obtained from subjects of various geographic locations in the intermingling way (Fig. 1). G3 was obtained only from Europeans as previously reported [3], and not from Asians. On the other hand G4, G5 and G6 were obtained mainly from Asians in this study. After bootstrap analysis for evaluation of the statistical reliability of the tree, the G1, G2, G3, G4, G5 and G6 clusters exhibited 99.2%, 85.7%, 98.5%, 98.3%, 99.3% and 99.0% reliability, respectively. The genetic distances between the six genotypes were very great, namely, 0.46217–0.80993 between G1 and G2, 0.41622–0.55145 between G1 and G3, 0.39398–0.70522 between G1 and G4, 0.61209–0.67842 between G1 and G5, 0.52042–0.65308 between G1 and G6, 0.26115–0.36384 between G2 and G3, 0.35794–0.56704 between G2 and G4, 0.50353–0.52332 between G2 and G5, 0.45225–0.55083 between G2 and G6, 0.40857–0.48626 between G3 and G4, 0.45536–0.52125 between G3 and G5, 0.39449–0.45657 between G3 and G6, 0.49698–0.65266 between G4 and G5, 0.57783–0.65898 between G4 and G6, and 0.57334–0.64124 between G5 and G6.

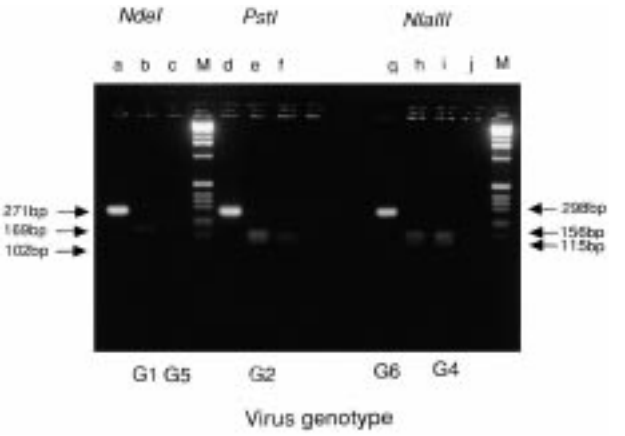

Fig. 3. Identification of restriction patterns obtained by restriction endonuclease digestion. Lanes a, b, c: RFLP pattern after *Nde*I digestion of the ORF 1 PCR products. Digestion of G1 with *Nde*I resulted in 169- and 102-bp fragments (lane b) and digestion of G5 resulted in 183- and 88-bp fragments (lane c). Lanes d, e, f: RFLP pattern after *Pst*I digestion of the ORF 1 PCR products. Digestion of G2 with *Pst*I resulted in 147- and 124-bp fragments (lane e) and another digestion pattern was found (lane f). TTV isolates, which were digested by neither *Nde*I nor *Pst*I, belonged to G4 or G6. It was possible to distinguish between G4 and G6 using restriction digestion by *Nla*III, which yielded 156- and 115-bp fragments (lanes g–j).

On the basis of these sequence data, restriction enzyme sites useful for determining each TTV genotype were identified by computer analysis; *Nde*I, *Pst*I, *Nla*III, and *Mse*I were selected and used for RFLP analysis (Fig. 2). Fig. 3 shows the restric-

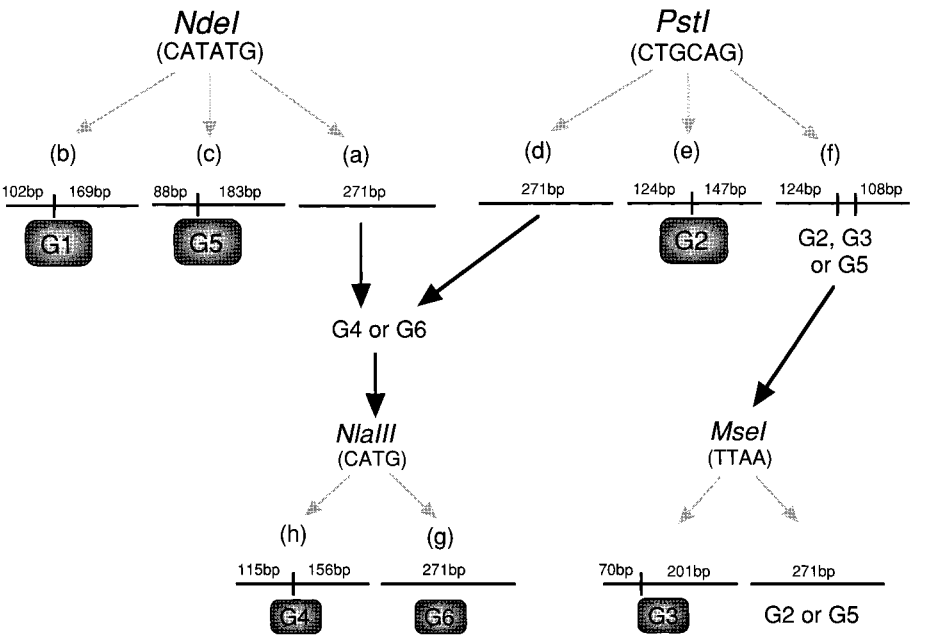

Fig. 4. The strategy for RFLP analysis with four restriction enzymes. Using the enzymes *Nde*I and *Pst*I, followed by cleavage with *Nla*III or *Mse*I, it was possible to distinguish between the six TTV genotypes. Digestion patterns indicated by a–h correspond to electropherotypes a–h in Fig. 3.

tion digestion pattern with each of these enzymes. The specific restriction site for *Nde*I was found at position 2016 (location of AB008394) in the amplified region for only the G1 isolates; digestion of G1 with *Nde*I resulted in 169- and 102-bp fragments. However, there was no restriction enzyme site for *Pst*I in any of the G1 isolates (Figs. 2 and 3). In G2, the specific restriction site for *Pst*I was found at position 2038 in the amplified region for all the isolates within this genotype, and hence these isolates were digested. There was no restriction site for *Nde*I in any of the G2 isolates (Figs. 2 and 3). When the PCR products of TTV were digested using both *Nde*I and *Pst*I, it was possible to distinguish between the two major TTV genotypes G1 and G2. As shown in Fig. 3, there was one isolate that showed a pattern of digestion with *Nde*I different from G1 isolates, which characteristically yielded 183- and 88-bp fragments. The specific restriction site for *Nde*I was found at position 2002 (Fig. 2) and phylogenetic analysis indicated that it belonged to G5 (Fig. 1). In this study, the other TTV isolates, which could be digested by neither *Nde*I nor *Pst*I, belonged to G4 or G6 as determined by phylogenetic analysis (Fig. 1). In G4, the specific restriction site for *Nla*III was found at position 2070 (Fig. 2), and hence it was possible to distinguish between G4 and G6 by *Nla*III digestion resulting in 156- and 115-bp fragments (Fig. 3). Recently, G3 isolates were obtained from Europe [3] and their nucleotide sequences were almost 80% identical to those of a part of G2. Interestingly, they had two restriction sites for *Pst*I at positions 2038 and 2077 (Fig. 2). In the sequences of G3, there existed a specific restriction site for *Mse*I which was not found in G2 (Fig. 2); however, we did not detect any G3 isolates.

Fig. 4 shows the final strategy for RFLP analysis used by us for TTV genotyping; using the enzymes *Nde*I and *Pst*I, followed by cleavage with *Nla*III or *Mse*I, it was possible to distinguish between the six TTV genotyes. Hence, the pattern of digestion by restriction enzymes allowed the assignment of specific genotypes to these 93 TTV isolates.

4. Discussion

A new single-stranded DNA virus designated TTV is often detected in patients with fulminant hepatitis, chronic cryptogenic liver diseases and hemophilia. This virus has also been detected in healthy blood donors [2,3,8]. As recently reported, the probability of detecting TTV infection increased with the severity of hemophilia and/or the amount of clotting factor treatment received. However, the prevalence among patients with hemophilia receiving exclusively heat-treated factor VIII has been reported to be very low (1.5%) [3]. These findings indicate that TTV is likely to be transmitted by blood and blood products not subjected to heat treatment for viral inactivation.

Of the known animal single-stranded DNA viruses, human parvoviruses such as parvovirus B19 have been suspected repeatedly as being the cause of non-A, non-B hepatitis in humans [9,10], although most do not cause hepatitis. TTV has several different genotypes as does hepatitis C virus (HCV) [2,3,8], and so there probably are specific TTV genotypes causing severe liver diseases or other diseases, although it still remains unclear whether TTV is a direct cause of disease.

In this study, we demonstrated the existence of three novel genotypes, G4, G5, and G6, other than the three previously reported genotypes of TTV [3]. Using two different strategies, molecular phylogenetic analysis by the N-J method and UPGMA (data not shown), six major clusters were obtained, and the high reliability of the six clusters was confirmed by bootstrap analysis. The genetic distances between the six genotypes of TTV were as great as those between the genotypes of HCV. The major genotypes 1a and 1b of HCV strongly correlate with the geographic distribution; genotype 1a is distributed in the United States and Europe, and genotype 1b is mainly distributed in Asia including Japan. In regard to TTV, the prevalence of G1 and G2 is very high worldwide, and these are probably major genotypes of TTV. The distribution of the major TTV genotypes, G1 and G2, was not related to their geographic distribution. This suggests that TTV, a single-stranded DNA virus, probably spread all over the world a long time ago and coexisted with humans for long without pathogenicity. To elucidate the evolution of TTV, it is necessary to construct phylogenetic trees using a greater number of TTV isolates worldwide and to examine if the other non-A to G agents are more closely related to TTV. Moreover, determination of nucleotide substitution rates will provide useful information regarding the evolution of TTV.

Another significant finding in this study was the design of a genotyping assay based on RFLP analysis for distinguishing between the six TTV genotypes. As the distribution of the different TTV genotypes might have potentially important clinical and epidemiological implications, it is necessary to evaluate the association of particular genotypes of TTV with the severity of liver diseases, viral quantification or response to interferon, as has been done for HCV. The genotyping of TTV is important; however, to identify the genotype of TTV, determination of the DNA sequence of each isolate is needed. We have developed a very simple genotyping method using four restriction enzymes (*Nde*I, *Pst*I, *Nla*III, *Mse*I), which recognize genotype-specific sites in the product by PCR; six genotypes can easily be identified by RFLP analysis. To confirm the genotyping assay based on RFLP analysis, furthermore, we examined more 145 isolates consisting of 100 G1, 40 G2 and 5 G4 isolates determined by phylogenetic analysis, and the results of genotyping by RFLP analysis were consistent with the results of phylogenetic analysis (data not shown). Certainly, the usefulness of this assay will need to be confirmed in prospective studies with TTV isolates from various geographical locations. If this approach is confirmed to be useful in larger studies, this simple method might be useful for an epidemiological survey of TTV. It may also be useful for studying the transmission of this virus in selected cases.

Finally, we did not find any relationship between liver diseases and TTV genotypes in this study. Our RFLP analysis, using a larger number of TTV isolates obtained from patients with some disease worldwide, might be useful for the discovery of certain disease-specific TTV genotypes.

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